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## A high-throughput robotic sample preparation system and HPLC-MS/MS for measuring urinary anatabine, anabasine, nicotine and major nicotine metabolites\*

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### Abstract

**Background**—Most sample preparation methods characteristically involve intensive and repetitive labor, which is inefficient when preparing large numbers of samples from population-scale studies.

**Methods**—This study presents a robotic system designed to meet the sampling requirements for large population-scale studies. Using this robotic system, we developed and validated a method to simultaneously measure urinary anatabine, anabasine, nicotine and seven major nicotine metabolites: 4-Hydroxy-4-(3-pyridyl)butanoic acid, cotinine-N-oxide, nicotine-N-oxide, trans-3'-hydroxycotinine, norcotinine, cotinine and normicotine. We analyzed robotically prepared samples using high-performance liquid chromatography (HPLC) coupled with triple quadrupole mass spectrometry in positive electrospray ionization mode using scheduled multiple reaction monitoring (sMRM) with a total runtime of 8.5 min.

**Results**—The optimized procedure was able to deliver linear analyte responses over a broad range of concentrations. Responses of urine-based calibrators delivered coefficients of determination ( $R^2$ ) of  $>0.995$ . Sample preparation recovery was generally higher than 80%. The robotic system was able to prepare four 96-well plate (384 urine samples) per day, and the overall method afforded an accuracy range of 92–115%, and an imprecision of  $<15.0\%$  on average.

**Conclusions**—The validation results demonstrate that the method is accurate, precise, sensitive, robust, and most significantly labor-saving for sample preparation, making it efficient and practical for routine measurements in large population-scale studies such as the National Health and Nutrition Examination Survey (NHANES) and the Population Assessment of Tobacco and Health (PATH) study.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cca.2014.06.012>.

## Keywords

Robotic; High-throughput; Biomonitoring; Biomarker; Tobacco exposure; Urinary metabolites

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## 1. Introduction

Humans are exposed to complex chemicals from both natural and anthropogenic sources that exist in both ambient surroundings and indoor microenvironments through daily inhalation, ingestion and dermal absorption. Tobacco smoke, for example, contains more than 8000 chemicals [1], many of which are associated with adverse health outcomes, such as cancer, respiratory and cardiovascular diseases, in both smokers and nonsmokers [2–5].

Assessing the exposure to and risk from chemicals caused by tobacco use by external measurements, e.g. their indoor air concentrations and surface loadings, is always challenging. Biomonitoring, measuring trace levels of suspected chemicals in biological matrices (i.e., blood, urine, and tissues), is able to provide the actual absorbed levels of these chemicals in human fluids and tissues, providing the “gold standard” for assessing exposure to chemicals [6].

However, biological samples are usually complicated matrices, containing thousands of chemicals from external exposure, their metabolites, and a number of other endogenous constituents, such as proteins, phospholipids and salts, which could potentially interfere with the analysis. As a result, biological samples require specific pre-treatment procedures to minimize or eliminate potential interferences and matrix effects and thus to improve the sensitivity and specificity for measuring the target analytes [7,8]. Typical pre-treatment techniques include liquid–liquid extraction (LLE) [9,10], solid phase extraction (SPE) [11–13], soxhlet extraction [14], supercritical fluid extraction (SFE) [15], microwave-assisted extraction (MAE) and pressurized solvent extraction (PSE) [16]. The robotic sample preparation procedure described here uses a validated acetone precipitation step to deplete endogenous proteins, salts and phospholipids and to remove the exogenous enzyme that is added to the urine samples to hydrolyze conjugates [17].

Target analytes in pretreated samples are often chromatographically resolved using gas chromatography (GC) or high-performance liquid chromatography (HPLC), which is often coupled with detectors varying from the ultraviolet (UV)/visible spectroscopy and single quadrupole mass spectrometry (MS) [10,18] to tandem MS/MS [11,19–21]. MS/MS provides a more sensitive and selective means for simultaneously measuring multiple analytes.

Most sample preparation methods characteristically involve intensive and repetitive labor, which is inefficient when preparing large numbers of samples from population-scale studies. These types of studies include the National Health and Nutrition Examination Survey (NHANES) designed to assess the health and nutritional status of adults and children in the United States [22], and the Population Assessment of Tobacco and Health (PATH) study aimed to assess the behavioral, social, and health impact of tobacco use [23]. The need for high through-put sample analysis motivated the development of a robotic procedure for

pretreatment of biological urine samples, specifically for measurement of anatabine (ANAT), anabasine (ANAB), nicotine (NIC) and seven major nicotine metabolites in the present study.

Nicotine, a primary tobacco-specific alkaloid in tobacco and tobacco smoke, does not directly cause most diseases associated with tobacco use. However, due to its addictiveness, users often choose to continuously/repeatedly use tobacco products, exposing themselves to the carcinogens and bioactive compounds in tobacco [24]. Thus, measuring nicotine and its major metabolites can determine tobacco exposure, and will play an important role in assessing tobacco exposure and making control regulations. Nicotine is absorbed by smokers mainly through direct inhalation of the mainstream smoke [24]. Non-smokers also can be involuntarily exposed to second-hand smoke (SHS), or even third-hand smoke through inhalation, dermal absorption, and dust ingestion [25, 26]. Nicotine in mammals is metabolized predominantly into cotinine (COT) (Fig. S1), accounting for approximately 70–80% of NIC dose. COT is further converted through various metabolism pathways, (e.g., cytochrome P450), to form other metabolites, including trans-3'-hydroxycotinine (3HC, accounting for 40–49% of NIC dose), cotinine-N-oxide (CNO, 2–5%) and norcotinine (NorCOT, 1–2%), leaving approximately 22–32% total COT in urine. Another three major NIC metabolites include 1-(3-Pyridyl)-1-butanol-4-carboxylic acid (HPBA, 7–9%), nicotine-N-oxide (NNO, 4–7%), and nornicotine (NorNIC, 0.4–0.8%) [24].

Tobacco plants synthesize nicotine as well as minor tobacco alkaloids such as ANAT and ANAB. This pattern of alkaloids is characteristic of tobacco leaf and tobacco products. Tobacco use leads to concurrent exposure to NIC, ANAT, and ANAB, as well as the presence of measurable levels of biomarkers of exposure to these alkaloids [27]. Not surprisingly, ANAT and ANAB are variably glucuronidated [17] and excreted in the urine with elimination half-lives similar to those for nicotine and its metabolites. Thus the presence of biomarkers of exposure to NIC, ANAT, and ANAB has been validated as proving tobacco product use [27]. Conversely, nicotine replacement therapy uses pharmaceutical-grade nicotine that is essentially free of minor tobacco alkaloids; therefore subjects abstaining from tobacco use and undergoing nicotine replacement therapy have urinary ANAT and ANAB levels <2 ng/ml.[27] Based on these findings ANAT and ANAB are validated biomarkers for identifying non-compliance for participants using NIC replacement therapy (NRT) [17,27]. Our specific aim in this study was to develop and validate a robotic sample preparation method and a HPLC-MS/MS analytical method to simultaneously determine the urinary concentrations of NIC and seven major NIC metabolites in samples from NHANES and PATH studies including both “free” (non-conjugated) and “conjugated” (mostly glucuronide) forms. Our automated method was able to prepare four 96-well plates (384 samples) per day simultaneously with a sample preparation recovery higher than 80%. The method was optimized to provide a wide linear dynamic range for analyte concentrations. Urine calibrators produced coefficients of determination ( $R^2$ ) of greater than 0.995. The accuracy, precision, and robustness make this method efficient and practical for large population-scale studies.

## 2. Materials and methods

### 2.1. Reagents and standards

Acetone, ammonium hydroxide, ammonium acetate and  $\beta$ -glucuronidase (type H-1, *Helix pomatia* and *Escherichia coli*, type IX-A) were from Sigma-Aldrich. Acetonitrile and methanol were from Honeywell; hydrochloric acid was purchased from Fisher Scientific and HPLC water was from J.T. Baker. Ten native analytes, including NIC, HPBA, CNO, NNO, 3HC, NorCOT, COT, NorNIC, ANAT and ANAB and their corresponding isotopically labeled standards were from Toronto Research Chemicals. Details for product sources are listed in Tables S1 and S2.

### 2.2. Biological samples

Urine samples from smokers ( $n = 40$ ) used to develop study methods were collected with no identifiable information by Tennessee Blood Services. Two hundred urine samples from non-smokers were collected from anonymous donors with Institutional Review Board (IRB) approval. Since the smoker urine samples were purchased from commercial sources, the analysis of these samples did not meet the definition of human subjects as specified in 45 CFR 46.102 (f) [28].

### 2.3. Blank urine pool preparation and Quality control (QC) materials

Blank urine used as matrix material for calibration standards and quality control (QC) was prepared using the following procedures: First, urine samples collected from non-users ( $n = 200$ ) were screened to eliminate those samples with detectable levels of the analytes. Then the samples containing non-detectable levels of the target analytes were pooled to form a blank urine pool and held at 4 °C overnight to ensure thorough mixing.

High and low QC pools made from the collected smoker urine samples ( $n = 40$ ) were prepared according to the following procedures: First, we screened smoker urine samples to determine the analyte concentrations in each sample; then, we combined different samples and diluted them using pooled blank urine to obtain a desired concentration for each analyte. It was necessary to spike some pools with native stock solutions to obtain the desired concentrations of some analytes in the pools when their concentrations did not yield high enough concentration levels. Aliquoted QC pools were analyzed daily for two months to obtain analyte means and standard deviations.

### 2.4. Standard preparation

Individual stock solutions were gravimetrically prepared for both native and labeled standards using certified materials in acidified HPLC water (0.1% hydrochloric acid in HPLC water). We prepared 12 calibration standard solutions by diluting the native stock solutions with pooled blank urine. We prepared internal standard spiking solution by mixing isotope-labeled stock solutions and diluting them with HPLC water. Details about the calibration ranges and isotope-labeled spiking internal standards are provided in Table 1.

## 2.5. HPLC mobile phase

Fresh mobile buffer “A” was prepared based on the volume needed for the total batch samples. For a total volume of 1.0 l buffer, we added 10 ml of 650 mmol/l of stock ammonium acetate solution to 990 ml of HPLC-grade water, yielding a running buffer of 6.5 mmol/l ammonium acetate. We adjusted the pH of the solution to 10.0 with ammonium hydroxide solution. Mobile phase “B” was 100% acetonitrile. Buffer solutions were degassed for 5 min using an ultrasonic water bath.

## 2.6. $\beta$ -Glucuronidase solution

Fresh enzyme solution (16.7 units/ $\mu$ l) was prepared by weighing 28.5 mg of  $\beta$ -glucuronidase (type H-1, *H. pomatia*) in 6.5 ml of 0.5 mol/l ammonium acetate (pH 5.1 adjusted with glacial acetic acid, stored at 4 °C), which resulted in 1000 units in 60  $\mu$ l solution. It was noted that enzyme solution should be mixed gently by inversion and prepared at least 30 min before use to ensure that all enzyme powder has dissolved.

## 2.7. Instrumentation

**2.7.1. Robotic sample preparation system**—The robotic sample preparation system was built in an enclosure (1.5 m deep, 2.3 m wide and 2.2 m high), containing a Mitsubishi robot, a Sciclone G3 automated liquid-handling workstation (PerkElmer), a Rotanda 460 auto-centrifuge, a thermal sealer (Thermo Scientific, USA), four Inheco incubators (Inheco), a TurboVap 96 concentration evaporator workstation (Biotage), a capper/de-capper (FluidX), and a 2D barcode reader (FluidX). Fig. 1 shows the scheme of the robotic sample preparation system. Additional information is provided in supporting materials, and more details can be obtained upon request.

**2.7.2. Analytic instruments**—We achieved chromatographic separation using a Gemini-NX column (100 mm  $\times$  3.0 mm, particle size 3  $\mu$ m, Phenomenex) on a HPLC system consisting of a DGU-20A5R degasser, two LC-20ADXR pumps, a SIL-30AC autosampler, and a CTO-20AC column oven (Shimadzu Corp.). The gradient system included 6.5 mmol/l of ammonium acetate (pH 10.0) (buffer A), and acetonitrile (organic phase) at a flow rate of 0.65 ml/min. We kept the column temperature at 40 °C during the entire analysis. Total chromatographic run time was 8.5 min; a sample chromatogram is shown in Fig. 2. Details about the HPLC gradient conditions are provided in Table 3S.

We performed tandem MS analysis on an ABSciex triple quadrupole 5500 mass spectrometer with a TurboIonSpray source (ABSciex). We operated the MS in electrospray (ESI) positive mode using scheduled MRM data acquisition. We monitored two MRM transitions for each analyte and one for the isotope labeled internal standard. The source/gas specific parameters applied in the method are listed in Table S4, and the analyte specific parameters and MRM transitions are provided in Table S5.

Analyst software (version 1.6.2) was employed for data acquisition, and data quantitation was performed using Indigo Ascent Automated Data Analysis and Review software — a web based integration software (Indigo Biosystems). Calibration curves were constructed

using peak area ratios of analytes to corresponding internal standards for each batch via linear least-squares regression with a  $1/x$  weighting factor.

## 2.8. Robotic sample preparation and cleanup principle

All sample preparation materials, including samples, plates and tips, etc., were automatically handled by the Mitsubishi robot, and samples were tracked throughout the process using the 2D barcode printed on each sample vial (Fig. 1). First, 50  $\mu$ l of internal standard solution (ISTD concentrations were listed in Table 1 and their sources were listed in Table S2) was transferred to each cell in a 96-deepwell plate. Then, 100  $\mu$ l of each sample (e.g., urine, QCs, calibration standards and laboratory control blanks) and 60  $\mu$ l enzyme solution were transferred to each cell in the 96-deepwell plate, and mixed well, followed by enzymatic hydrolysis at 45 °C for 12 h. After the plate was cooled to room temperature, 450  $\mu$ l cold acetone (−20 °C) was added to each well. The plate was held at −20 °C for 30 min followed by centrifugation at −20 °C for 30 min, and then 180  $\mu$ l of the supernatant in each well was transferred into a second 96-well plate and evaporated for about 12 min to remove acetone. Finally, 250  $\mu$ l HPLC water was added into each well prior to HPLC injection. Details of the sample preparation on the automation system are provided in the supporting materials.

## 2.9. Quality control program

Following quality control (QC) measures were used to ensure the reliability of the data: 1). calibration standards (STD) and QC samples were kept at or below −60 °C for long-term storage; 2). STDs, QCs and laboratory control blanks were prepared and analyzed in the same manner as the urine samples in each analytical batch; 3). Samples were calibrated using 12-point curves, and both calibration standards and QC samples were prepared in pooled urine samples to correct for potential matrix effects; 4). Calibration curves were regularly assessed using standard solutions prepared by spiking chemicals from a second commercial source or lot in nonsmoker urine pools; 5). Instruments were regularly evaluated to maintain high sensitivity. Specifically, the mass spectrometer source was cleaned weekly, usually on Monday of each week, and the MS tuning was routinely conducted semi-annually. But any maintenance was also done on an as-needed basis or before an unusual low response for any of the analyte was seen; 6). The data were quantified using Indigo customized for this method for automatic peak selection and integration. Specifically, following rules were customized for this method to ensure the data quality, including ion ratio (qualitative peak area/quantitative peak are), thresholds such as blank contamination, extreme concentration, calibration linearity, standard concentration deviation, instrumental sensitivity, QC concentration range, retention time, carry over, and maximum instrument intensity; and 7). The QC program of the Division of Laboratory Sciences, National Center for Environmental Health (NCEH) at CDC, were used to evaluate the accuracy and precision of the analyte concentrations in QC samples [29].

## 3. Results and discussion

The robotic sample preparation system we present in the study provides a technically feasible means to avoid extensive and repetitive manual work during routine biological sample preparation required for large numbers of samples from population-scale studies



(e.g. NHANES and PATH). We applied this system further to determine ANAT, ANAB, NIC and its seven major metabolites in human urine using a modified method based on the study by McGuffey et al. [17]. We conducted the modifications aimed to increase the throughput using the robotic sample preparation system, mainly in 2 aspects: Liquid chromatography (column, buffer and gradient) and enzymatic hydrolysis. To ensure the reliability of the automated system when applied to the method, we then evaluated the sensitivity, linearity, accuracy, precision, sample preparation recovery, sample stability (light, temporal and thermal) and carry-over.

### 3.1. Mobile phase and liquid chromatography

Several major factors influencing chromatography, e.g. buffer pH and gradient, were tested to ensure that we achieved the best resolution and reduced the analysis time. Those factors are not independent but mutually related. In this study, we selected a Gemini-NX column with moderate dimension ( $100 \times 3.0$  mm,  $3.0 \mu\text{m}$ ) and adjusted the gradient to obtain a running time of 8.5 min per sample (Table 2). Meanwhile, we reduced HPLC injection volume to  $2 \mu\text{L}$  to avoid overload and detector saturation. The flow rate was set to  $0.65 \text{ mL/min}$ . Responses of all analytes on the detector and their resolutions were compared at different buffer pH values from 3.5 to 10.5 as presented in Fig. 3.

Most previous studies used acidic buffers in their methods for measuring urinary NIC and its metabolites [12,21]. As reported in those studies and indicated in Fig. 3, many analytes overlap under acidic conditions. Although quantitating the analytic concentrations using different MRM transitions was possible, the following factors could cause large uncertainties and variations in calculated concentrations: first, NIC and many of its metabolites always share the same MRM transitions that give the highest peak responses. To differentiate analytes, different MRM transitions should be selected for each analyte. Inevitably, some MRM transitions with lower peak response must be selected for certain analytes, which will lead to lower sensitivities for those analytes. Second, biological samples are always complicated matrices. Potential interferences that have the same MRM transitions and retention times can distort the peak shapes and deteriorate the chromatograph baseline, resulting in larger uncertainties and variations in peak picking and integrating. Finally, low resolutions from analyte to analyte and from analytes to interferences may also increase the ion suppression, and further reduce the sensitivity.

However as shown in Fig. 3, basic buffer ( $\text{pH} > 9.6$ ) can significantly improve the chromatographic resolutions. A buffer with higher pH greater than 10.0 (e.g. 10.5) can reduce the peak tailing to some extent, especially for NIC, NorNIC, ANAB and ANAT as the column ages (typically  $>1200$  runs). Using this method, the retention time for the first eluted analyte, HPBT, slowly shifted to the void volume after 800–1200 runs, the usual retention time in which polar matrix materials are eluted. Consequently, ion suppression gradually increased for HPBA, which required replacing the old column before heavy tailing issues occurred. To consider the theoretical pH range for ammonia–ammonium acetate buffer (8.2–10.2) and the instrument pH tolerance, to maximize the column life and the method ruggedness, and to delay the elution time of HPBA from polar waste, we chose a buffer pH of 10.0 in this method.

### 3.2. Enzymatic hydrolysis

Hydrolysis is a critical step to “free” the analyte from all conjugated forms, such as glucuronide (Fig. S1) to obtain the “total” concentration for each analyte. We tested two types of enzymes, *E. coli* and *H. pomatia*, and evaluated their hydrolysis performances by changing the enzyme amount added to each sample at three incubation temperatures: 37°, 45° and 50 °C (Figs. 4 and S2). For those 10 analytes with same amount added to each sample at 37° and 45 °C, *E. Coli* generally has higher activity than *H. Pomatia*. However, with a temperature increase from 45° to 50 °C, the activity of *E. Coli* slowly decreases while the activity for *H. Pomatia* slowly increases. Thus, the usable working incubation temperature range for *H. Pomatia* is wider than that for *E. Coli*. Usually with the same conditions for other parameters, hydrolysis takes longer to complete at 37 °C than at 45 °C for both enzymes. To allow completion of the enzymatic hydrolysis within 12 h, we selected 45 °C as the incubation temperature.

Acid hydrolysis (hydrochloric acid) performance was also tested to investigate whether acid hydrolysis can typically reduce the incubation duration. However, we found that the hydrochloric acid did not complete the hydrolysis for urinary 3HC-O- $\beta$ -D-glucuronide within even a longer time, indicating that the efficacy of acid hydrolysis could be associated with the specific analyte. In addition, a liquid–liquid extraction or SPE cleanup is generally necessary after acid hydrolysis to remove the acid which is not compatible with the instrument. Those sample cleanup procedures are not as amenable to automation as the validated acetone precipitation procedure used with enzyme hydrolysis.

### 3.3. Sensitivity and linearity

Our preliminary studies showed that urinary concentration varied vastly from one analyte to another. 3HC, COT and NIC had the highest urinary concentrations that can exceed 12,000 ng/ml. Urinary concentrations for HPBA, CNO and NNO could be as high as 5000 ng/ml; the concentrations for NorCOT and Normic were typically lower than 1000 ng/ml. Urinary concentrations for ANAT and ANAB were on average lower than 100 ng/ml. Usually high concentrations can saturate the MS detector, while low concentrations may fall below the limit of detection (LOD). To determine all 10 analytes simultaneously, and to increase their dynamic linear ranges to reduce sample re-run rate, we detuned analytes with high concentrations (Table 3) and reconstituted processed samples with 250  $\mu$ l water prior to HPLC injection. We did not detune ANAT and ANAB to maintain their sensitivities because they have relatively low urinary concentrations. Typical linear equations with correlation coefficients ( $R^2$ ) that exceeded 0.995 are also presented in Table 1.

### 3.4. LODs and LOQs

The LOD and the limit of quantitation (LOQ) for all analytes were determined by preparing and analyzing four low spiked urine pools. The standard deviation (SD) of each pool (Y axis) was plotted against the concentration (X axis). The estimate of the SD at zero analytic concentration ( $S_0$ ) was defined as the Y-intercepts. LODs and LOQs were calculated as three times and 10 times  $S_0$  [30–32], respectively, as given in Table 1. Notably, the sensitivities for those detuned chemicals were inevitably lower compared with those sensitivities under optimized conditions. Since this method was developed to evaluate urine



samples from smokers and persons heavily exposed to SHS, those LOQs were sufficient to obtain high detection rates.

### 3.5. Accuracy and imprecision

Intra- and inter-day accuracy (expressed as a percent of the target concentration) and imprecision for each analyte were evaluated with 5 different concentrations distributed across the entire calibration curves of measured analytes prepared by spiking known amounts of analytes in the pooled blank urine (Table S7). Specifically, inter-day accuracy and imprecision were evaluated with 16 replicates for each concentration level within an analytical batch each day. The inter-day data was assessed with three separate analytical batches over 3 consecutive days. The total accuracy and imprecision were calculated with all data sets ( $n = 48$ ) (Table 3). The overall intraday imprecision was  $< 10\%$ , except for a few samples at low concentrations, for which the imprecision was around 15%.

### 3.6. Sample preparation recovery and carry-over

Two sets of samples, including three replicates from each of the low, medium and high concentration pools, were prepared to determine the recovery for each analyte on the automated sample preparation and the potential carry-over on the HPLC-MS/MS system using a method similar to that used by McGuffey et al. [17]. Briefly, deuterated internal standard solution was added to one set of samples at the beginning of the sample preparation; the solution was added to the other set immediately before injection. Sample preparation recovery (%) was calculated by comparing the average peak area of processed internal standards with the average peak area of unprocessed internal standards.

Chemicals can be lost through procedures such as absorption, precipitation and evaporation during the entire sample preparation. Take the evaporation procedure for example, because of the variety of the volatility for those analytes included in the method, the loss fraction for each analyte during evaporation is different. Smoker urine samples usually have high analyte concentrations, which, in most cases, still allow enough mass amounts in prepared samples to be detected by MS even when the sample preparation recovery is low. However, lower recovery will simultaneously lead to lower detection rate, especially for those chemicals with lower urinary concentrations, such as anatabine and anabasine. Thus, these chemicals may not be detected even in light smokers. In this study, we strictly set the evaporation parameters to just remove the acetone that was added for precipitation so as to avoid drying the residuals. This process ensures acceptable detection rates for all analytes and good consistence for sample preparation. Average recovery measured for all analytes was greater than 87%. NNO and NorCOT had the 2 highest recoveries greater than 92%, and NIC had the lowest recovery of 80%. These results from the automation system are comparable to those reported in previous studies using the manual methods [12,21].

To investigate the carry-over, we used the same sample pools that were used for determining the sample preparation recovery. After the injection of each sample (low, median, high), three blank urine samples were injected. The carry-over was determined by comparing the average peak area of each native analyte in the first blank urine sample with the average peak area for the native analyte in those processed samples (low, median, high). Validation

results indicated that carry-over for each analyte was negligible at all levels (high, median and low).

### 3.7. Thermal, temporal, light and freeze–thaw stability

All samples were stored at or below  $-60^{\circ}\text{C}$ . Because the samples were prepared at room temperature and could be potentially exposed to light during processing, we evaluated thermal, temporal and light stability for each analyte (Figs. S3 and S4, supporting materials). These results indicated that all measured analytes were stable during the entire sample preparation procedures under laboratory conditions, including the 12-h incubation at  $45^{\circ}\text{C}$ , and were stable for at least 2 months at  $-20^{\circ}\text{C}$ . Processed samples were stable for at least 1 week in the liquid chromatography autosampler at  $4^{\circ}\text{C}$ .

Urine samples might undergo multiple freeze–thaw cycles when it becomes necessary to re-prepare or aliquot them. We tested two urine pools to measure whether this procedure could affect target analyte concentrations. The results indicated that no significant effects of freeze–thaw cycles (up to 25 cycles) were detected on the targeted analyte concentrations (Table S6). However, we suggest that all samples be appropriately capped to avoid any loss or condensation due to liquid evaporation during the freeze–thaw procedure.

**3.7.1. QC-charts**—Using the robotic system, we characterized and analyzed two QC pools (high and low) on 20 individual analytical batches using the optimized HPLC-MS/MS method for 12 consecutive weeks. QC charts for all analytes are shown in Figs. S5A and S5B, demonstrating excellent accuracy, precision and ruggedness of this method.

### 3.8. Application to smoker urine samples

We applied the fully validated method to measure the total concentrations (“free” plus “conjugated” forms) for those 10 analytes in a set of urine samples from smokers ( $n = 40$ ). Table 2 shows the concentration distributions for each target analyte. The highest values for both geometric mean (GM) and median were observed for 3HC (GM: 6236 ng/ml; median: 6695 ng/ml), followed by COT (GM: 3223 ng/ml; median: 3555 ng/ml). NIC had the third highest GM of 1178 ng/ml and median of 1485 ng/ml. Both GMs and medians for HPBA, CNO and NNO were lower than 1000 ng/ml and those for NorCOT and NorNIC were  $< 150$  ng/ml.

Estimated molar percentages of the summation of the molar concentrations for NIC and its metabolites are shown in Fig. 5. On average, 3HC accounted for 48% of the molar fraction in the total molar concentrations in this set of samples, followed by COT and NIC, accounting for 27% and 11%, respectively. Molar fractions for HPBA, CNO, NNO, NorNIC and NorCOT were 5%, 4%, 3%, 1% and 1%, respectively. These percentages were comparable to those observed results in metabolism studies in mammals [24].

Nicotine and its metabolites can be detected in urine samples from not only cigarette smokers but also from persons using nicotine replacement therapy with smoking cessation drugs (e.g. nicotine patch, gum, nicotine inhaler, and nasal spray). However, ANAT and ANAB predominantly exist in cigarette products and less frequently in smokeless products. A previous study suggested a cut-point of 2 ng/ml to differentiate cigarette smokers and

persons undergoing nicotine replacement therapy [27]. Using this cut-point, only two persons among those 40 samples had concentrations for both ANAB and ANAT <1.1 ng/ml, indicating that they might have been undergoing nicotine replacement therapy when the urine samples were collected. Five persons had concentrations around 2 ng/ml for ANAT and ANAB, which could have been caused by either light cigarette smoking or exposure to heavy SHS when the urine samples were collected.

## 4. Conclusions

In this study, we presented a fully automated sample preparation system that was able to circumvent the intensive and repetitive labor normally required for large numbers of samples from population-scale studies (e.g. NHANES and PATH). The automated system was able to simultaneously prepare four 96-well plate (384 urine samples) per day with a mean preparation recovery greater than 80%. A HPLC-ESI-MS/MS method was then used to measure anatabine, anabasine, nicotine and seven major nicotine metabolites in human urine. Wide calibration linear dynamic ranges can reduce the sample re-preparation rate significantly. Validation results demonstrate that this method is robust, accurate, precise, and efficient for preparing and analyzing routine urine samples.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

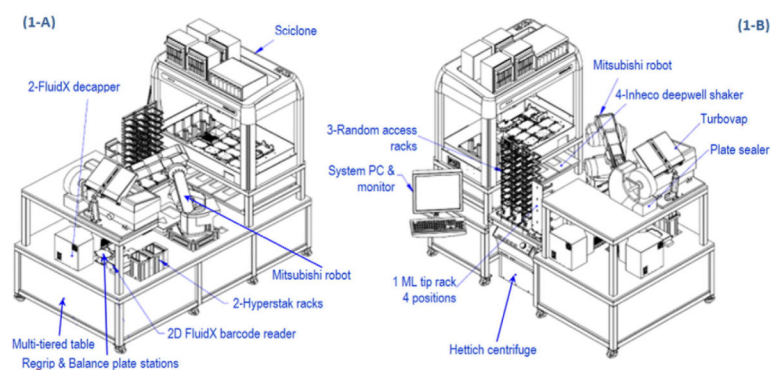
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## References

- [1]. Rodgman, A.; Perfetti, TA. The chemical components of tobacco and tobacco smoke. 2nd. CRC Press; New York: 2009.
- [2]. Otsuka R, Watanabe H, Hirata K, Tokai K, Muro T, Yoshiyama M, et al. Acute effects of passive smoking on the coronary circulation in healthy young adults. JAMA. 2001; 286(4):436–41. [PubMed: 11466122]
- [3]. Chilmonczyk BA, Salmun LM, Megathlin KN, Neveux LM, Palomaki GE, Knight GJ, Rodgman A, et al. Association between exposure to environmental tobacco smoke and exacerbations of asthma in children. N Engl J Med. 1993; 328(23):1665–9. [PubMed: 8487825]
- [4]. Hopkin J, Evans H. Cigarette smoke-induced DNA damage and lung cancer risks. Nature. 1980; 283:388–90. [PubMed: 7352014]
- [5]. US-CDC. Health effects of secondhand smoke. 2013. Available from: [http://www.cdc.gov/tobacco/data\\_statistics/fact\\_sheets/secondhand\\_smoke/health\\_effects/](http://www.cdc.gov/tobacco/data_statistics/fact_sheets/secondhand_smoke/health_effects/) [Access date: Jan. 21, 2014]
- [6]. Sexton K, Needham L, Pirkle J. Human biomonitoring of environmental chemicals measuring chemicals in human tissues is the “gold standard” for assessing people's exposure to pollution. Am Sci. 2004; 92:38–45.
- [7]. Matuszewski B, Constanzer M, Chavez-Eng C. Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per milliliter concentrations. Anal Chem. 1998; 70(5):882–9. [PubMed: 9511465]

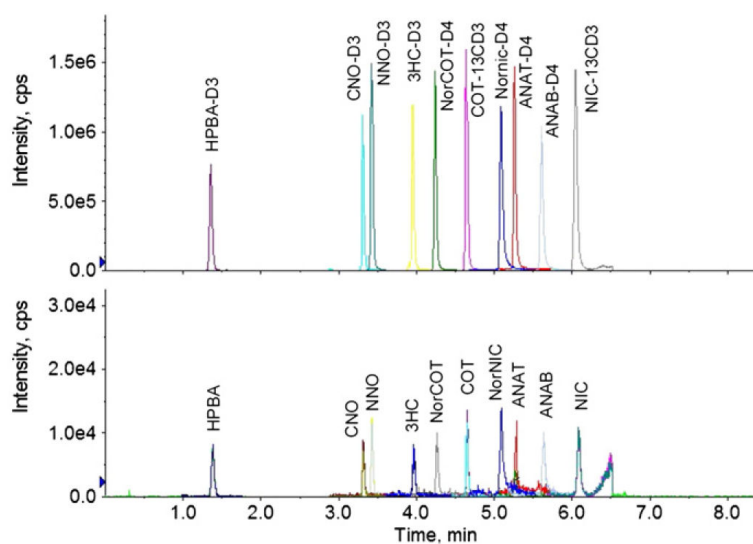
- [8]. Liang H, Foltz R, Meng M, Bennett P. Ionization enhancement in atmospheric pressure chemical ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2003; 17(24):2815–21. [PubMed: 14673832]
- [9]. Jacob P, Havel C, Lee DH, Yu L, Eisner MD, Benowitz NL. Subpicogram per milliliter determination of the tobacco-specific carcinogen metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in human urine using liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2008; 80(21):8115–21. [PubMed: 18841944]
- [10]. Wei B, Mohan KR, Weisel CP. Exposure of flight attendants to pyrethroid insecticides on commercial flights: Urinary metabolite levels and implications. *Int J Hyg Environ Health*. 2012; 215(4):465–73. [PubMed: 21937269]
- [11]. Bernert JT, Turner WE, Pirkle JL, Sosnoff CS, Akins JR, Waldrep MK, et al. Development and validation of sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography atmospheric pressure ionization tandem mass spectrometry. *Clin Chem*. 1997; 43(12):2281–91. [PubMed: 9439445]
- [12]. Xu X, Iba MM, Weisel CP. Simultaneous and sensitive measurement of anabasine, nicotine, and nicotine metabolites in human urine by liquid chromatography-tandem mass spectrometry. *Clin Chem*. 2004; 50(12):2323–30. [PubMed: 15472033]
- [13]. Scheidweiler KB, Shakleya DM, Huestis MA. Simultaneous quantification of nicotine, cotinine, trans-3'-hydroxycotinine, norcotinine and mecamlamine in human urine by liquid chromatography-tandem mass spectrometry. *Clin Chim Acta*. 2012; 413(11):978–84. [PubMed: 22394455]
- [14]. Seidel V, Lindner W. Universal sample enrichment technique for organochlorine pesticides in environmental and biological samples using a redesigned simultaneous steam distillation–solvent extraction apparatus. *Anal Chem*. 1993; 65(24):3677–83.
- [15]. Hawthorne SB. Analytical-scale supercritical fluid extraction. *Anal Chem*. 1990; 62(11):633A–42A.
- [16]. Kaufmann B, Christen P. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. *Phytochem Anal*. 2002; 13(2):105–13. [PubMed: 12018022]
- [17]. McGuffey JE, Wei B, Bernert JT, Morrow JC, Xia B, Wang L, et al. Validation of a LC-MS/MS method for quantifying urinary nicotine, six nicotine metabolites and the minor alkaloids – anatabine and anabasine – in smokers' urine. *PLoS One*. 2014 in press.
- [18]. Chambers DM, McElprang DO, Waterhouse MG, Blount BC. An improved approach for accurate quantitation of benzene, toluene, ethylbenzene, xylene, and styrene in blood. *Anal Chem*. 2006; 78(15):5375–83. [PubMed: 16878872]
- [19]. Feng J, Wang L, Dai I, Harmon T, Bernert JT. Simultaneous determination of multiple drugs of abuse and relevant metabolites in urine by LC-MS-MS. *J Anal Toxicol*. 2007; 31(7):359–68. [PubMed: 17725883]
- [20]. Alwis KU, Blount BC, Britt AS, Patel D, Ashley DL. Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS). *Anal Chim Acta*. 2012; 750:152–60. [PubMed: 23062436]
- [21]. Miller EI, Norris HRK, Rollins DE, Tiffany ST, Wilkins DG. A novel validated procedure for the determination of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine by solid-phase extraction coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry. *J Chromatogr B*. 2010; 878(9):725–37.
- [22]. CDC. National Health and Nutrition Examination Survey (NAHNES). 2013. Available from: <http://www.cdc.gov/nchs/nhanes.htm> [Access date: January 21st, 2014]
- [23]. US-FDA/NIH. Population Assessment of Tobacco and Health — the PATH study. 2013. Available from: <https://pathstudyinfo.nih.gov/UI/Home.aspx> [Access date: January 21st, 2014]
- [24]. Hukkanen J, Jacob P, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev*. 2005; 57(1):79–115. [PubMed: 15734728]

- [25]. Vardavas CI, Fthenou E, Patelarou E, Bagkeris E, Murphy S, Hecht SS, et al. Exposure to different sources of second-hand smoke during pregnancy and its effect on urinary cotinine and tobacco-specific nitrosamine (NNAL) concentrations. *Tob Control*. 2013; 22(3):194–200. [PubMed: 22253001]
- [26]. Helen G, Bernert JT, Hall DB, Sosnoff CS, Xia Y, Balmes JR, et al. Exposure to second-hand smoke outside of a bar and a restaurant and tobacco exposure biomarkers in nonsmokers. *Environ Health Perspect*. 2012; 120(7):1010–6. [PubMed: 22484462]
- [27]. Jacob P, Hatsukami D, Severson H, Hall S, Yu L, Benowitz NL. Anabasine and anatabine as biomarkers for tobacco use during nicotine replacement therapy. *Cancer Epidemiol Biomarkers Prev*. 2002; 11(12):1668–73. [PubMed: 12496059]
- [28]. US-HHS. U.S. Department of Health & Human Services. Human subjects research (45 CFR 46): Code of Federal Regulations. 2010. Available from: <http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.html> [Access date: Jan. 21, 2014]
- [29]. Caudill SP, Schleicher RL, Pirkle JL. Multi-rule quality control for the age-related eye disease study. *Stat Med*. 2008; 27(20):4094–106. [PubMed: 18344178]
- [30]. DNR. Analytical detection limit guidance & laboratory guide for determining method detection limits. 1996. Available from: <http://dnr.wi.gov/regulations/labcert/documents/guidance/-LODguide.pdf> [Accessed date: Jan. 22, 2014]
- [31]. ICH. ICH harmonised tripartite guideline. Validation of Analytical Procedures: Text and, Methodology. 1994.
- [32]. Taylor, JK. Quality assurance of chemical measurements. CRC Press; 1987.

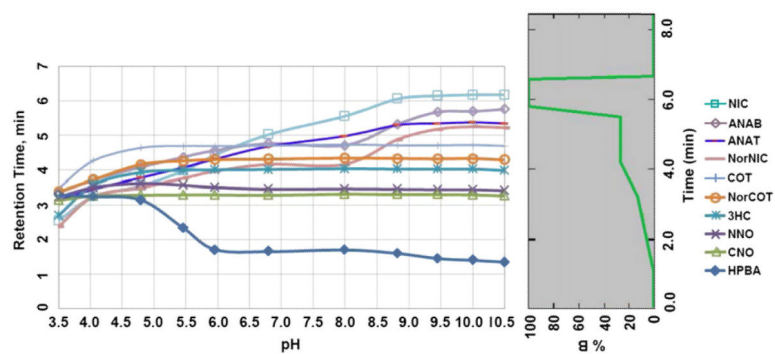


**Fig. 1.**  
The scheme for the robotic sample preparation system (back side-1A, front side-1B).

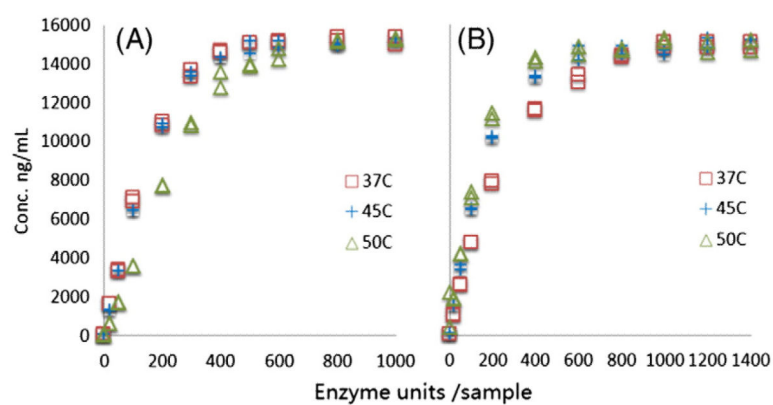


**Fig. 2.**

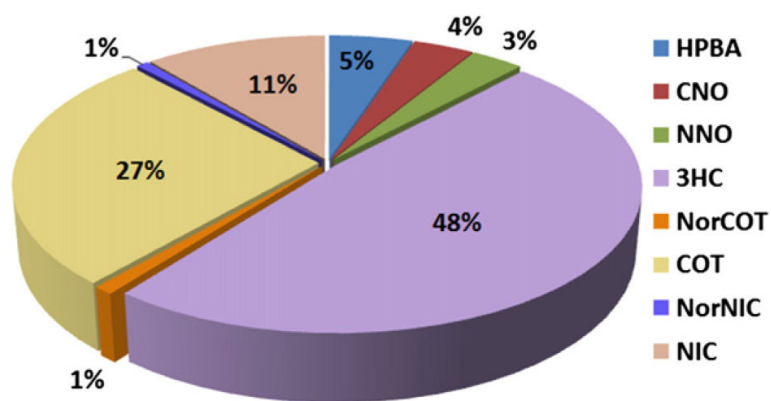
A representative chromatograph for a urine sample (HPBA: 3.50; CNO: 6.0; NNO: 8.0; 3HC: 25; NorCOT: 3.0; COT: 15; NorNIC: 3.0; ANAT: 1.0; ANAB: 0.80; and NIC: 20. Unit: ng/ml).



**Fig. 3.**  
Effects of buffer (A) pH on the retention time for each analyte assessed using the running gradient in Table S3.



**Fig. 4.** Enzymatic hydrolysis performances between *E. Coli* (A) and *H. Pomatia* (B) for Urinary 3HC-O-β-D-glucuronide (critical glucuronide determining the enzyme amount and incubation duration) at 37, 45 and 50 °C.



**Fig. 5.**  
Estimated median molar percentages for nicotine metabolites over the total molar concentrations.

**Table 1**

Native calibration range, Isotope-labeled internal standard concentration (ISTD), typical calibration equation, limit of detection (LOD) and limit of quantitation (LOQ) (unit: ng/ml).

Analyte	ISTD conc. ng/ml	Calibration range ng/ml	Calibration equation <sup>a</sup>	<i>R</i> <sup>2</sup>	LOD	LOQ
HPBA	1326	1.0–6000	$y_{\text{response\_ratio}} = 6.9556\text{e} \text{ (—4)} \cdot x_{\text{conc}} + 3.5874\text{e} \text{ (—4)}$	0.99926	0.40	1.40
CNO	456	1.5–4500	$y_{\text{response\_ratio}} = 6.1846\text{e} \text{ (—4)} \cdot x_{\text{conc}} - 84057\text{e} \text{ (—5)}$	0.99924	0.60	2.00
NNO	304	2.0–6000	$y_{\text{response\_ratio}} = 4.0443\text{e} \text{ (—4)} \cdot x_{\text{conc}} + 2.0414\text{e} \text{ (—4)}$	0.99973	0.75	2.50
3HC	1290	3.0–55,000	$y_{\text{response\_ratio}} = 7.5744\text{e} \text{ (—5)} \cdot x_{\text{conc}} + 8.9021\text{e} \text{ (—7)}$	0.99986	2.85	9.50
NorCOT	726	0.75–1600	$y_{\text{response\_ratio}} = 2.1099\text{e} \text{ (—3)} \cdot x_{\text{conc}} + 7.6984\text{e} \text{ (—4)}$	0.99997	0.33	1.10
COT	344	1.5–25,000	$y_{\text{response\_ratio}} = 9.7285\text{e} \text{ (—5)} \cdot x_{\text{conc}} + 7.0885\text{e} \text{ (—5)}$	0.99995	1.40	4.60
NorNIC	571	0.0–1200	$y_{\text{response\_ratio}} = 2.5500\text{e} \text{ (—3)} \cdot x_{\text{conc}} + 2.8787\text{e} \text{ (—2)}$	0.99994	0.75	2.50
ANAT	367	0.25–800	$y_{\text{response\_ratio}} = 4.1524\text{e} \text{ (—3)} \cdot x_{\text{conc}} + 5.3714\text{e} \text{ (—4)}$	0.99975	0.12	0.40
ANAB	507	0.20–800	$y_{\text{response\_ratio}} = 3.6507\text{e} \text{ (—3)} \cdot x_{\text{conc}} + 4.1881\text{e} \text{ (—3)}$	0.99983	0.15	0.50
NIC	5474	0.0–20,000	$y_{\text{response\_ratio}} = 1.3560\text{e} \text{ (—4)} \cdot x_{\text{conc}} + 7.6751\text{e} \text{ (—4)}$	0.99999	3.15	10.5

<sup>a</sup>Response-ratio: native response divided by ISTD response;  $x_{\text{conc}}$ : sample concentration, ng/ml.

**Table 2**

Concentration distributions (ng/ml) for anatabine, anabasine, nicotine and major nicotine metabolites in 40 smoker urine samples.

Analyte	GM	Min	5th	25th	50th	75th	95th	Max
HPBA	636	44.2	110	397	685	1140	2205	3070
CNO	501	53.9	121	297	573	807	1540	2790
NNO	408	26.2	65.4	208	469	724	2474	3230
3HC	6236	1010	1642	3893	6695	11125	18,785	30,800
NorCOT	111	15.8	27.3	75.0	117	175	368	482
COT	3224	267	1106	2175	3555	5383	8080	21,200
NorNIC	74.2	5.31	11.5	50.0	80.3	148	365	468
ANAB	16.2	0.80	1.97	10.2	17.7	39.5	69.8	73.8
ANAT	8.61	0.26	1.73	5.47	10.1	18.9	33.8	38.3
NIC	1178	28.1	108	623	1485	3395	6071	7110



**Table 3**

Overall accuracy and precision measured during a period of three months (n = 48).

	Target	Measured	STD	RSD%	Error%		Level-1	Level-2	Level-3	Level-4	Level-5	Measured	STD	RSD%	Error%
HPBA	Level-1	8.00	8.33	0.38	4.73	4.09	COT					40.7	1.79	4.47	1.76
	Level-2	40.0	41.7	1.66	4.15	4.18		Level-2				205	6.85	3.42	2.48
	Level-3	320	334	11.7	3.66	4.39		Level-3				1637	42.2	2.64	2.30
	Level-4	1600	1671	55.2	3.45	4.44		Level-4				8162	276	3.45	2.02
	Level-5	4000	4229	113	2.83	5.71		Level-5				20,527	676	3.38	2.64
CNO	Level-1	8.00	8.19	0.49	6.10	2.37	NorNIC	Level-1				2.06	0.17	8.33	2.89
	Level-2	40.0	39.8	1.43	3.57	-0.56		Level-2				10.3	0.67	6.73	2.53
	Level-3	320	321	8.59	2.68	0.33		Level-3				82.8	2.65	3.31	3.46
	Level-4	1600	1598	37.1	2.32	-0.10		Level-4				411	14.4	3.60	2.85
	Level-5	4000	4016	112	2.79	0.41		Level-5				1051	47.6	4.76	5.15
NNO	Level-1	8.00	8.14	0.34	4.31	1.70	ANAT	Level-1				1.22	0.06	5.10	1.89
	Level-2	40.0	40.0	1.42	3.56	-0.01		Level-2				6.07	0.24	3.99	1.09
	Level-3	320	320	9.61	3.00	-0.01		Level-3				50.2	1.61	3.36	4.59
	Level-4	1600	1618	36.8	2.30	1.12		Level-4				251	9.10	3.79	4.47
	Level-5	4000	4085	110	2.75	2.13		Level-5				616	18.3	3.05	2.64
3HC	Level-1	80.0	80.8	2.25	2.82	0.98	ANAB	Level-1				1.03	0.13	13.0	3.38
	Level-2	400	397	11.9	2.98	-0.73		Level-2				4.97	0.23	4.53	-0.64
	Level-3	3200	3149	85.1	2.66	-1.60		Level-3				39.7	2.44	6.09	-0.83
	Level-4	16,000	15778	258	1.61	-1.39		Level-4				192	7.77	3.88	-4.11
	Level-5	40,000	39347	995	2.49	-1.63		Level-5				487	16.2	3.23	-2.65
NorCOT	Level-1	2.20	2.10	0.11	5.01	-4.44	NIC	Level-1				30.5	1.38	4.60	1.68
	Level-2	11.0	10.3	0.40	3.65	-6.73		Level-2				151	4.27	2.85	0.72
	Level-3	88.0	83.3	2.95	3.35	-5.36		Level-3				1207	37.4	3.11	0.61
	Level-4	440	417	12.1	2.75	-5.35		Level-4				6086	135	2.25	1.43
	Level-5	1100	1044	35.3	3.21	-5.12		Level-5				15,321	231	1.54	2.14